REFEREED

ORIGINAL RESEARCH

Evaluation of Plasmid Delivery by Electroporation as a Means of Increasing Gonadotropin-Releasing Hormone Production in Stallions

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ABSTRACT

A plasmid delivery system validated in other species was assessed for its potential for inducing long-term expression of gonadotropin-releasing hormone (GnRH) in stallions. The efficacy of this technique was demonstrated using two plasmids: pSEAP, expressing secreted embryonic alkaline phosphatase (SEAP), and pGnRH, expressing GnRH. In experiment 1, geldings were used as a model to test the effect of muscle of injection (splenius, pectoralis, and semitendinosus; n = 3 for each site) on the expression of the reporter plasmid, pSEAP. Concentrations of SEAP rose ($P < .01$) in jugular plasma samples, indicating uptake and expression of the pSEAP plasmid. Concentrations of SEAP were greatest ($P < .05$) and most consistent after pectoralis injection, and this site was chosen for injection and electroporation in the subsequent experiment. In experiment 2, stallions were treated with pGnRH (2 mg, n = 3; and 4 mg, n = 3) or 2 mg of pSEAP (control; n = 4) to determine the effects on the reproductive axis. Treatment with pGnRH (day 0) resulted in higher ($P < .05$) plasma testosterone concentrations from day 35 to 56 and increased the luteinizing hormone (LH) ($P < 0.01$) and testosterone ($P < .1$) responses to GnRH challenge on day 21. Daily semen characteristics from days 31 to 36 showed no effect ($P > .1$) of pGnRH treatment on seminal characteristics. It was concluded that delivery by electroporation of plasmids encoding peptide hormones may serve as a means of long-term in vivo production of peptides in the horse. Increases in LH and testosterone secretion after GnRH were observed in pGnRH-treated stallions; however, optimal conditions for expression need to be determined in future experiments.

Keywords: Electroporation; GnRH; Plasmid; Reproduction; Stallion

INTRODUCTION

Increasing the libido and sperm output of older, valuable stallions, or of stallions in general during the winter months, could increase the number of breedings and reduce the labor input in a particular breeding season. Gonadotropin-releasing hormone (GnRH) therapy has been tested as a treatment for improving gonadotropin and testosterone secretion in these scenarios. Chronic, pulsatile administration of GnRH to stallions[1] increased luteinizing hormone (LH) and testosterone secretion but was less effective on seminal characteristics. Pulsatile GnRH therapy is generally impractical because of the labor needed and the long-term nature of the treatment. Alternatively, administration of long-lasting, potent GnRH analogs initially increases gonadotropin concentrations but subsequently down-regulates gonadotropin production.[2,3] The aim of the current experiment was to develop a novel, plasmid-mediated delivery system for extrahypothalamic expression of GnRH. Delivery of plasmids with promoters optimized for mammalian muscle tissue and the use electroporation, a process by which the permeability of the plasma membrane of surrounding cells is temporarily increased by an externally applied electrical field to allow entry of large molecules, has resulted in high expression levels of protein in mice,[4,5] rats,[6,7] dogs,[8,9] and pigs.[10,11]

An initial experiment was designed to determine the effect of muscle of injection on plasmid expression of a "reporter" protein (secreted embryonic alkaline phosphatase; SEAP) not found in adult horses. A specific plasmid was then made that included a muscle-specific promoter followed by a cDNA coding for the first 33 amino acids of the porcine GnRH gene.[12] The plasmid was designed for long-term expression of GnRH, with the goal of increasing

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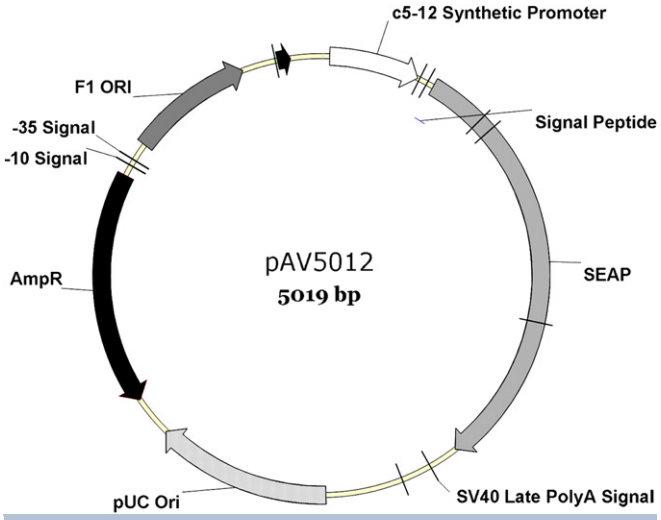

Figure 1. Map of the plasmid encoding for secreted embryonic alkaline phosphatase (pSEAP). The 5019-base pair circular plasmid contains the c5-12 synthetic promoter and the cDNA for SEAP. The plasmid is used extensively as a reporter for in vivo and in vitro studies.

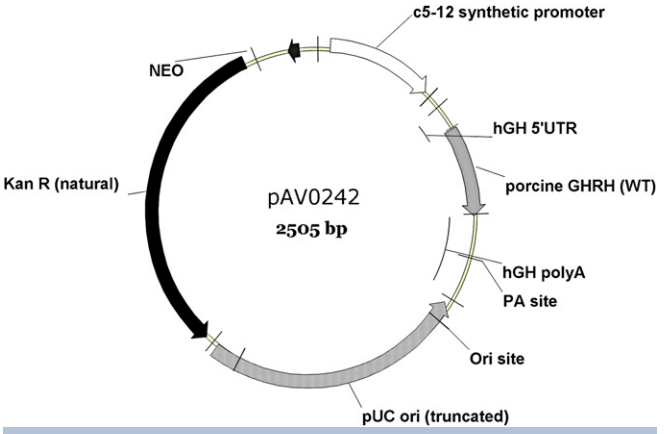

Figure 2. Map of growth hormone releasing hormone plasmid (pGHRH) used as the starting point for the GnRH-encoding plasmid used in experiment 2. The 2,505–base pair circular plasmid contains the c5-12 synthetic promoter and the cDNA for GHRH, which was replaced with a cDNA encoding for the first 33 amino acids of porcine GnRH.

production of gonadotropins and testosterone in stallions. If successful, such technology would likely have similar application in seasonally anestrous mares.

MATERIALS AND METHODS

Experiment 1. Assessment of Muscle Groups for Plasmid Delivery

Nine light-horse geldings, 5 to 15 years of age, weighing between 500 and 600 kg with body condition scores[13] of 6 to 8, were maintained on native grass pasture with supplemental grass hay as needed to maintain body condition. On day 0 (June 22, 2005), three geldings received delivery by electroporation of a muscle-specific plasmid expressing SEAP (pSEAP, Fig. 1; VGX Pharmaceuticals, Immune Therapeutics Division, Woodlands, TX) into the body of one of three muscles: splenius in the neck, pectoralis in the chest, and semitendinosus in the rump.

In preparation for injection of the plasmid and electroporation, horses were first sedated with 1.1 mg/kg xylazine and 0.02 mg/kg butorphanol administered intravenously to effect; they remained standing throughout the procedure. The delivery site was clipped and sanitized with chlorhexidine. Subsequently, the electrode needle array portion of an electroporation device, the CELLECTRA™ IM array (VGX Pharmaceuticals, The Woodlands, Texas) was inserted. The IM array consists of a mounted circular array (1 cm diameter) of five evenly spaced, 21-gauge solid stainless steel electrodes (2 cm in length) mounted on a nonconductive plastic disc. After insertion of the IM array, the plasmid was injected intramuscularly via a 21-gauge needle into the center of the needle array. Eighty seconds after plasmid injection, a constant current was delivered at 0.5 amperes, 3 pulses, 52 milliseconds each, with 1 second between pulses. The plasmid was delivered into the selected muscle site as 2 mg DNA in 2 mL sterile water for injection + 1% poly-L-glutamate (wt/wt).

Jugular blood samples were collected via venipuncture on days 0 (before injection), 3, 7, 10, 14, 17, 21, 24, 27, and 30 relative to treatment. Blood samples were immediately centrifuged (1,200*g* at 5°C for 15 minutes) after collection; plasma was harvested and frozen at -15°C until assay. Plasma from daily samples was analyzed for SEAP by chemiluminescent assay (Phospha-Light System, Applied Biosystems, Bedford, MA).

Experiment 2. Effects of Plasmid Encoding for GnRH in Stallions

The plasmid designed for this experiment (pGnRH) was generated from a muscle-specific plasmid encoding porcine growth hormone releasing hormone[14] (GHRH). The plasmid was reconstructed by replacing the existing GHRH cDNA sequence (Fig. 2) with the cDNA encoding GnRH by enzymatic cleavage at the 5' Nco I and 3' Hind III sites. A new cDNA insert was constructed for alternative expression of GnRH based on the porcine gene for GnRH[12] (GeneBank accession #L32864). The constructed insert coded for the first 33 amino acids of the porcine GnRH cDNA were modified to include an Nco I restriction site at the 5′ end and two 3′ stop codons in conjunction with a Hind III restriction site. The

translated protein from this construct included the signal peptide and the mature 10–amino acid porcine GnRH. The complete cDNA insert was generated by polymerase chain reaction (PCR) from two overlapping primer templates: [5′-CCATGGAGCCAATTCCGAAACTTCTAGCC GGACTTCTGCTGCTG**ACTCTGTGTGTAGTGGGCTG C**-3′ (65 bp) and 5′-AAGCTTTCATTATCCAGGGCGCA ATCCATAGGACCAGTGTTGGCTGGA**GCAGCCCACTA CACACAGAGT**-3′ (69 bp)] and extended with primers designed for annealing at the generated 5′ and 3′ ends [5′-CCATGGAGCCAATTCCGAAA-3′ (20 bp) and 5′- AA GCTTTCATTATCCAGGGCG-3′ (21 bp)]. The resulting PCR product was cloned using the TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA). Colonies were selected by kanamycin resistance, plasmid DNA was purified via QIAfilter Plasmid Midi Kit (Qiagen Inc., Valencia, CA), and the sequence was verified. Both the TOPO-XL vector and the pGHRH plasmid were simultaneously double-digested with restriction endonucleases Nco I and Hind III (Invitrogen) for 24 hours at 37°C. The digested plasmid and insert products were then separated by agarose gel electrophoresis and purified with the PureLink Gel Extraction Kit (Invitrogen). The products were ligated using the DNA Ligation Kit Ver.1 (Takara Mirus Bio Inc., Madison, WI). The resulting plasmid was transfected into a DHS alpha *Escherichia coli* cell line and selected based on kanamycin resistance. Plasmid DNA was purified from resulting colonies with QIAfilter Plasmid Midi Kit (Qiagen) and the sequence verified by PCR. Select colonies were grown and plasmid DNA was purified with EndoFree Plasmid Giga Kit (Qiagen).

Treatment of Stallions

Ten stallions ranging in age from 2 to 25 years were used. They had seminal characteristics within normal ranges; however, no fertility data were available for them. They were allotted to three groups such that average age and weight were similar for the groups. On day 0 (June 15, 2006), stallions in the first group (n = 3) received intramuscular injection and electroporation of 2 mg pGnRH in 2 mL sterile water for injection + 0.1% poly-L-glutamate; stallions in the second group (n = 3) received 4 mg pGnRH in 2 mL of the same vehicle; and those in the third group (n = 4) received 2 mg pSEAP in 2 mL vehicle as described for experiment 1 (these served as controls for the pGnRH-treated stallions). Preparation and sedation of the stallions were performed as described for experiment 1; injection and electroporation of the plasmids was in the pectoralis muscle of the chest. All stallions were treated on the same day.

Blood samples were collected from all stallions twice weekly beginning 1 week before treatment and continuing through week 6 after treatment. On day 21, stallions were fitted with an indwelling jugular catheter. One hour later, all stallions received a challenge injection of GnRH (0.1 μg/kg body weight, intravenously; Sigma Chemical Co., St. Louis, MO), and blood samples were collected at −20, −10, 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 minutes relative to injection to assess the pituitary-gonadal response to GnRH. These blood samples were analyzed for concentrations of LH, follicle-stimulating hormone (FSH), and testosterone.

Semen collection was conducted daily for 6 days starting on day 31 after plasmid injection. Semen evaluation[15] was conducted on the last three ejaculates from each stallion. Gel volume, gel-free volume, progressive motility, concentration, and sperm morphology were evaluated. Morphological characteristics (head, midpiece, and tail abnormalities and proximal and distal droplets) were assessed in gel-free semen fixed in 2% buffered formol-saline; 100 sperm from each ejaculate were viewed under phase contrast microscopy.[16]

Blood collected during frequent and daily sampling was immediately centrifuged (1,200*g* at 5°C for 15 minutes), and plasma was harvested and stored at -15°C until assay. All samples were analyzed as previously described for SEAP (Phospha-Light System, Applied Biosystems); LH,[17] FSH,[18] and testosterone[19] were measured by radioimmunoassays previously validated for equine plasma.

Data in each experiment were analyzed for effects of treatment and time and the treatment by time interaction by one-way analysis of variance (ANOVA) with repeated measures using the Mixed Model mixed procedure of SAS (SAS Institute Inc., Cary, NC). Differences at individual time points were determined by Least Significant Difference test (SLICE command in SAS) when a significant F ($P < .05$) was detected. Plasma concentrations of LH, FSH, testosterone, and SEAP were calculated as net differences from pretreatment means for each individual before ANOVA to account for individual variation in pretreatment, resting hormone concentrations.

RESULTS

Given the sedation of the geldings and stallions, only minor reaction was observed to the procedures. There were generally local twitches from insertion of the needle array, and slight startle responses on occasion when electroporation was in progress. Visual appraisals of the injection/electroporation sites were conducted in both experiments. Minor swelling was detected at the site in most of the horses in the first 24 hours; most swelling subsided by 24 hours, and none was detected at 48 hours or later.

Experiment 1

Concentrations of SEAP increased ($P < .01$) in jugular plasma after injection and electroporation of the SEAP-expressing plasmid, pSEAP, averaged over all 3 muscle sites. There was also an interaction between muscle site

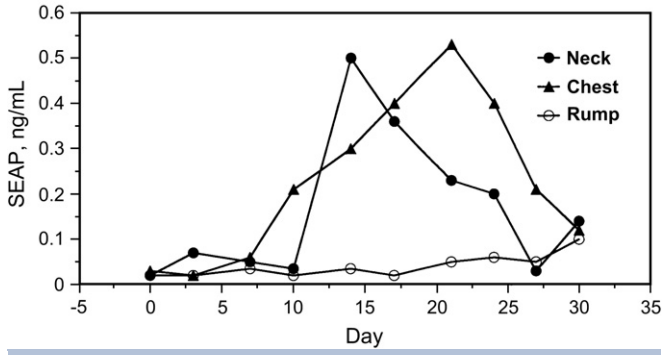

Figure 3. Mean plasma concentrations of SEAP for samples collected twice weekly in response to injection and electroporation with pSEAP (time 0) in the neck (splenius), chest (pectoralis), or rump (semitendinosus). Concentrations of SEAP increased over time ($P < .01$) and were greatest ($P < .05$) and most consistently high when delivered in the chest. The pooled SEM was 0.11 ng/mL.

and time, with the greatest ($P < .05$) response seen in geldings that received the plasmid injection into the pectoralis muscle of the chest (Fig. 3).

Experiment 2

Responses for the 2- and 4-mg pGnRH injection did not differ ($P > .1$); thus, the groups were combined and referred to collectively as "pGnRH-treated." Plasma testosterone concentrations in pSEAP-treated stallions (Fig. 4) gradually decreased from the onset of treatment through day 56; treatment with pGnRH resulted in higher ($P < .05$) plasma concentrations of testosterone relative to pSEAP-treated stallions by day 35 after treatment, which persisted through day 56. Plasma concentrations of LH and FSH in samples collected twice weekly did not differ ($P > .1$) between groups (Fig. 4). Frequent blood sampling conducted around the GnRH challenge on day 21 revealed that pGnRH treatment increased the LH ($P < .01$) and testosterone ($P = .098$) responses to GnRH relative to pSEAP-treated stallions (Fig. 5); the FSH response was not altered ($P > .1$).

Control stallions responded to pSEAP injection with increased ($P < .01$) plasma concentrations of SEAP (Fig. 6). Seminal characteristics did not differ ($P > .1$) between groups or across days (Fig. 7).

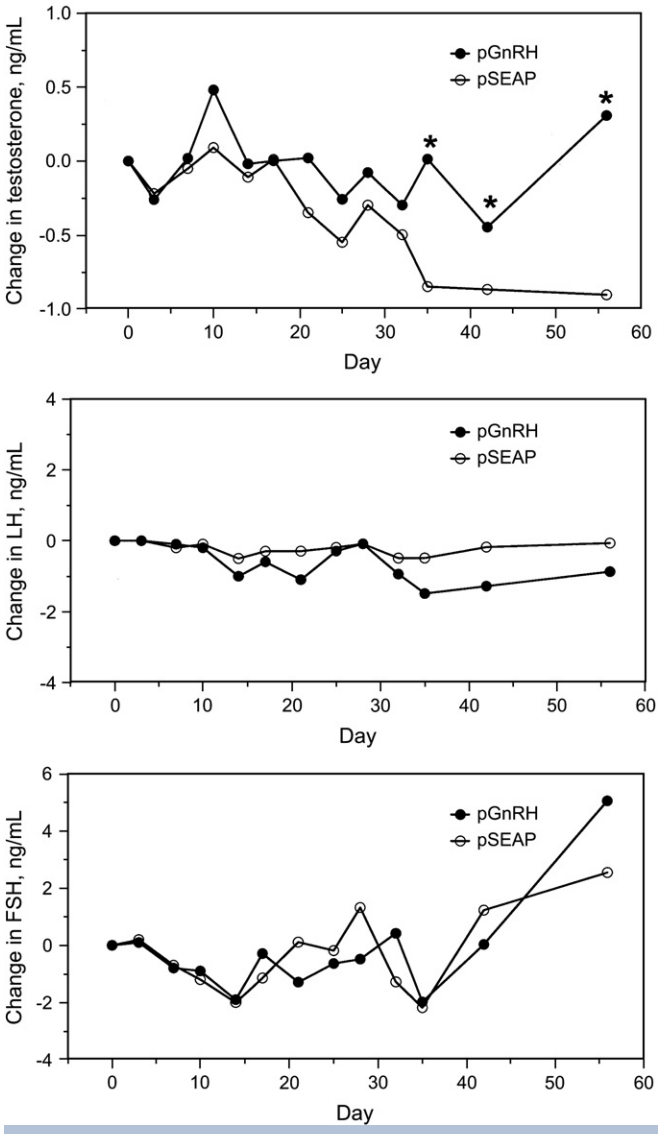

Figure 4. Mean plasma concentrations, expressed as net change from pretreatment, for testosterone, LH, and FSH in samples collected twice weekly after pGnRH (2- and 4-mg groups combined) and pSEAP treatment on day 0. Testosterone concentrations were higher ($P < .05$) in pGnRH-treated stallions relative to pSEAP-treated stallions on day 35 and thereafter. Concentrations of LH and FSH were similar between groups. Pooled SEM were 0.33, 0.46, and 0.81 for testosterone, LH, and FSH, respectively. Asterisks indicate differences between groups ($P < .05$).

DISCUSSION

The pSEAP plasmid was chosen for experiment 1 because it was known to be efficacious in other species,[20,21] and it provided an easily measurable protein product not normally found in adult stallions. In experiment 1, intramuscular plasmid injection followed by electroporation proved to be an effective delivery system for the pSEAP plasmid in the horse. As indicated by assay of peripheral SEAP concentrations, intramuscular electroporation was efficacious. Nevertheless, pSEAP was differentially expressed, or taken up, in the three muscles. Circulating

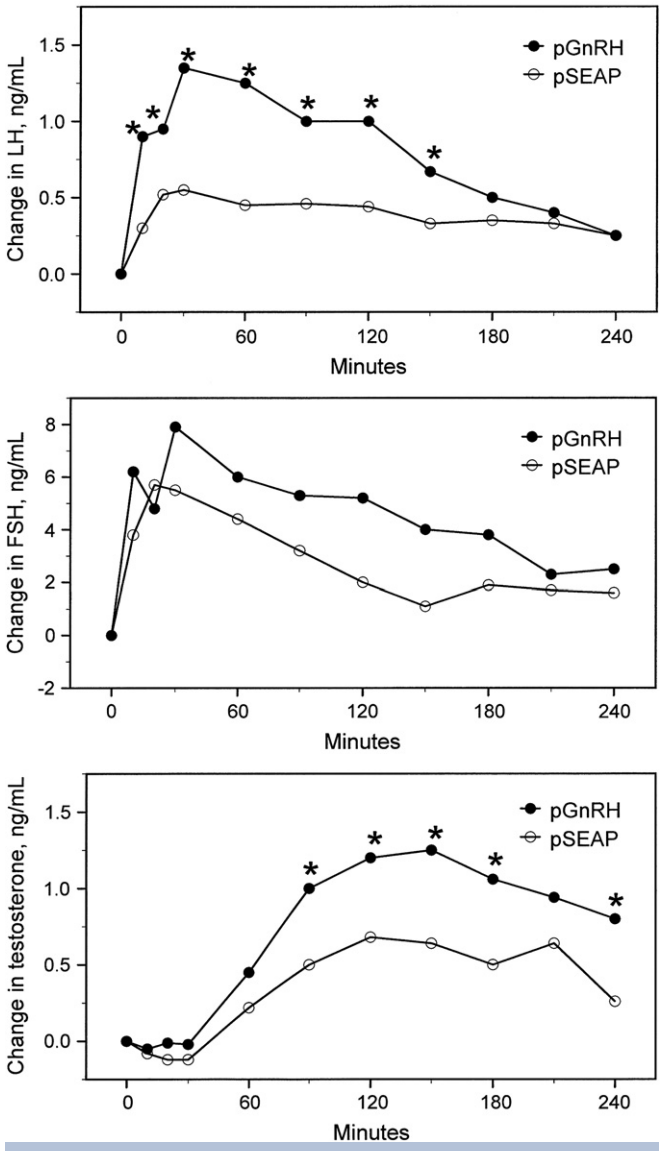

Figure 5. Plasma concentrations, expressed as net change from pre-injection, for LH, FSH, and testosterone in pGnRH (2- and 4-mg groups combined) and pSEAP-treated stallions after GnRH administration (time 0) on day 21. Stallions treated with pGnRH had a greater response in concentrations of LH ($P < .05$) and testosterone ($P < .1$); concentrations of FSH were similar between groups. Pooled SEM were 0.22, 3.8, and 0.24 ng/mL for LH, FSH, and testosterone, respectively. Asterisks indicate differences between groups for LH ($P < .05$) and testosterone ($P < .1$).

SEAP levels after plasmid electroporation were reliable and consistent when the plasmid was delivered into the pectoralis muscle. The lower expressions of SEAP in geldings treated in the splenius and semitendinosus muscles were

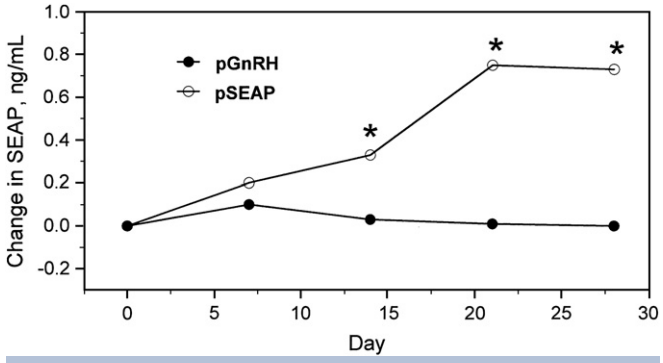

Figure 6. Plasma concentrations of SEAP in pGnRH (2- and 4-mg groups combined) and pSEAP-treated stallions expressed as individual difference from pretreatment means. Treatment with pSEAP increased ($P < .05$) plasma concentrations of SEAP. Pooled SEM was 0.15 ng/mL. Asterisks indicate differences between groups ($P < .05$).

also associated with a greater variation among individual subjects. Efficacy of the treatment and accessibility of the pectoralis make it an ideal treatment site for application of this technique in horses. Thus, the pectoralis muscle was chosen as the site for injection and electroporation in experiment 2.

SEAP, being foreign to the adult horse, is recognized as such and is cleared by the immune system[21] in a timely manner. This results in a sharp, transient increase in plasma concentrations of SEAP (with a peak expression at days 14 to 21 after electroporation) followed by a similarly sharp decrease and return to baseline. In pigs, electroporation of a plasmid encoding the SEAP cDNA resulted in peak plasma SEAP concentrations by day 10 before beginning to decline.[11,22]

Treatment of stallions with the plasmid encoding for GnRH resulted in higher testosterone concentrations from day 35 through the end of the sampling period (day 58). The gradual drop in testosterone concentrations in pSEAP-treated stallions was likely attributable to normal seasonal changes, given the time of year the experiment was conducted. The possibility that SEAP production in those stallions injected with pSEAP caused a drop in testosterone production and secretion cannot be ruled out; however, there is no evidence to our knowledge that short-term SEAP exposure, or the clearance of SEAP by the immune system, would result in a decrease in testosterone secretion by the testes. Blue and colleagues[1] reported an increase in testosterone secretion in stallions in response to long-term, pulsatile administration of GnRH (10 μg every 2 hours). Blue et al[1] also reported that continuous infusion of GnRH at a similar rate was not effective in increasing testosterone or LH secretion. The exact nature of GnRH expression from pGnRH in this experiment is unknown,

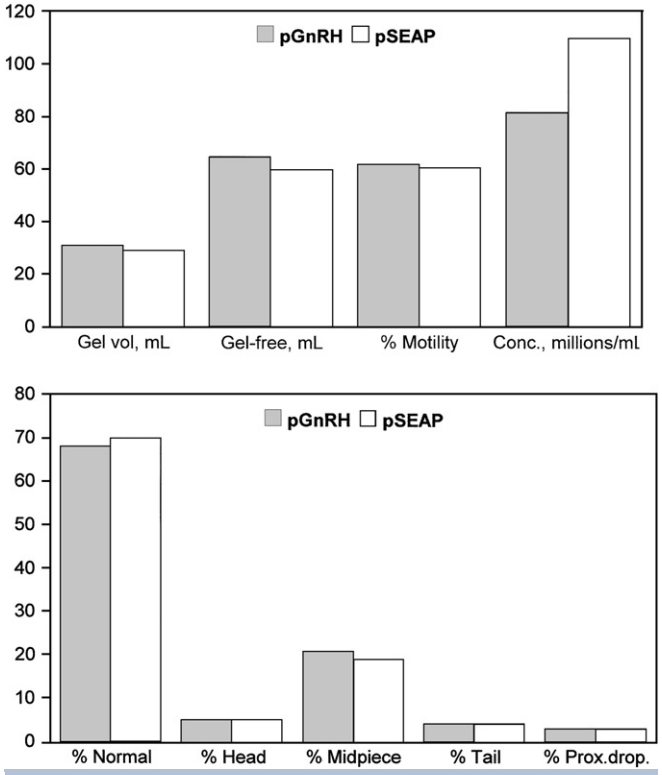

Figure 7. Seminal characteristics of stallions treated with pGnRH (2- and 4-mg groups combined) or pSEAP on day 0. Semen was collected from all stallions daily from day 31 to 36; data from the last three ejaculates were used for analysis. There was no effect of treatment or day of collection ($P > .1$) on seminal characteristics. Pooled SEM were 9 and 10 mL for volumes of gel and gel-free semen, 15% for percent motility, 20 million/mL for sperm concentration, and 11, 1, 10, 2, and 0.5% for percent normal and percent head, midpiece, tail, and droplet abnormalities, respectively.

although it is likely to be relatively continuous, or constant, as opposed to the episodic or pulsatile nature of endogenous GnRH secretion in most species.[23,24]

Treatment with pGnRH was not effective in increasing basal (daily) concentrations of gonadotropins but did increase the LH response to a physiologic dose of GnRH on day 21. It is likely that increasing concentrations of testosterone provided negative feedback adequate to inhibit LH secretion from the pituitary[25] while production was still being stimulated by GnRH. In seasonally anovulatory mares,[26] daily injections of a GnRH analog potentiated the LH response to each consecutive injection, whereas basal LH concentrations between GnRH injections were similar to controls. This indicates an ability of GnRH to increase intracellular production of LH without noticeable increases in basal secretion. In the current experiment, intracellular production of LH may have been augmented at the same time that steroid inhibition was minimizing LH secretion. Intuitively, increased levels of testosterone should have been accompanied by a reduction in LH secretion. Maintenance of basal LH concentrations in the treated group, therefore, likely indicated a positive effect of pGnRH.

Horses have been reported to be fairly resistant to down-regulation by GnRH and its analogs. Boyle and colleagues[2] used osmotic pumps to deliver buserelin (a potent GnRH analogue) to assess the long-term effects of high levels of GnRH. In their study, LH and testosterone concentrations were initially increased and subsequently decreased below baseline. Based on those data, we suspect that the GnRH expression from pGnRH did not attain levels required for down-regulation. Current interest in the horse industry has included utilization of potent GnRH analogs for regulating reproduction-related behavior. Future efforts might be directed toward evaluating the effects of improved expression or incorporating genetic expression of GnRH analogs into the plasmid for treatment of reproduction-related behavior in the horse.

Seminal characteristics were not altered when semen was evaluated on days 34 through 36, in spite of the increased testosterone secretion. Blue et al.[1] similarly reported that constant infusion or pulsatile administration of GnRH for 5 months did not alter total number of spermatozoa or progressive motility. This may reflect the lack of perturbation in basal LH and FSH secretion in response to pGnRH treatment. Also, considerable differences in testosterone secretion were not evident until day 35. Differences attributed to the variation in testosterone secretion may have been evident if semen collection had been conducted later in the study.

Increased concentrations of SEAP in pSEAP-treated stallions confirmed the functionality of the technique in the stallion. As stated previously, SEAP concentrations may not adequately reflect the expression pattern of GnRH. The SEAP-encoding plasmid was used in experiment 1 to determine the ideal muscle to be treated and in experiment 2 as a control to confirm that the electroporation technique operated appropriately in the horse. Considering that the pGnRH was delivered with the same technique, in the muscle identified to elicit consistent and less variable transgene product levels, and that both expression cassettes were driven by the same muscle-specific promoter, it is assumed that GnRH was expressed similarly to SEAP. In contrast to SEAP, GnRH should not elicit an immune response because of its natural occurrence in the horse and its relatively small molecular weight. Thus, the duration of expression of pGnRH would potentially be much longer than that of pSEAP.

In conclusion, intramuscular plasmid delivery followed by electroporation seems to have potential as an effective hormone delivery system for the horse. The current experiments indicated that the basic components of the SEAP and GHRH[14] plasmids could be used to construct plasmids containing the DNA of other potentially beneficial peptides, such as GnRH. Increased testosterone production in the stallion was accompanied by a perturbation in LH secretion after GnRH challenge, providing indirect evidence that GnRH was expressed in response to pGnRH. Furthermore, the increase in testosterone production was not associated with adverse physiologic effects with regard to down-regulation of gonadotropins or semen morphology. These results warrant future research into the possible beneficial uses of this technology for the equine industry.

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